REMARKS

Applicant respectfully requests reconsideration. Claims 1-13 were previously pending in this application. Claim 13 is withdrawn from consideration. No claims have been amended or cancelled. As a result, claims 1-12 are pending for examination with claims 1, 6 and 12 being independent claims. No new matter has been added.

Rejection under 35 U.S.C. § 112

Claims 4-5 and 9-11 are objected to under 37 C.F.R. 1.75(c) as being in improper format because multiple dependent claims cannot depend on other multiple dependent claims.

Appropriate corrections have been made. Accordingly, reconsideration and withdrawal of this rejection is requested.

Rejection Under 35 U.S.C. 102

Claims 1-3, 6-8, and 12 are rejected under 35 U.S.C. 102(b) as being anticipated by Ghannadan et al. (Int Arch Allergy Immunol. 127: 299-307, 2002). The Examiner states that "Ghannadan et al. disclose a) contacting a biological sample of HNC-1 cell line with the mAb for endothelial cell C protein (CD201) and b) separating cells that bind to the mAb by FACS analysis thereby producing a substantially pure population of EPCR+ hemmatopoietic (sic) stem cells" (pages 2-3 of the Office Action). The claimed invention is not anticipated by Ghannadan et al.

There appears to be an error in this rejection and in the rejection under 35 U.S.C § 103: the Examiner characterizes Ghannadan et al. as disclosing contacting a biological sample of HNC-1 cell line. However, Ghannadan et al. does not disclose the use of any HNC-1 cell line; instead, Ghannadan et al. describes the use of HMC-1 cell line. Applicant assumes that the Examiner intended to refer to HMC-1 cell line and will address the rejections accordingly.

An anticipatory reference must teach each and every limitation of a claim. Ghannadan et al. does not teach a method for obtaining a substantially pure population of hematopoietic stem cells as instantly claimed. The HMC-1 cell line is an immature mast cell line derived from a patient with mast cell leukaemia (Butterfield JH et al. Leuk Res. 1988;12(4):345-55; copy enclosed). The HMC-1 cell line does not produce hematopoietic stem cells. It would not have been possible to obtain

hematopoietic stems cells from the HMC-1 cell line. Ghannadan et al. examined the expression of CD antigens on human mast cells and basophils using monoclonal antibodies and found that the endothelial cell C protein antibody had a 26-46% reactivity with the mast cell line HMC-1 (see Abstract, Introduction sections on pages 299-300 and Table 2). The HMC-1 cell line is described as having many characteristics of immature mast cells and being useful for the study of mast cells and their constituents (Butterfield JH et al. Leuk Res. 1988;12(4):345-55; copy enclosed). Mast cells are highly specialized cells found resident in tissues throughout the body and play a role in allergy and anaphylaxis (Metcalfe DD et al. Physiol Rev. 1997 Oct;77(4):1033-79; cited in the Information Disclosure Statement filed herewith). Hematopoietic stem cells are a rare population of bone marrow cells with the capacity to reconstitute the entire hematopoietic system. The mast cell line does not produce hematopoietic stem cells and, therefore it would not have been possible to obtain hematopoietic stem cells using HMC-1 and an endothelial cell C protein antibody; Ghannadan et al. does not disclose a method by which hematopoietic stem cells can be obtained or a method by which a substantially pure population of hematopoietic stem cells can be obtained. Ghannadan et al. does not anticipate the pending claims.

Accordingly, reconsideration and withdrawal of the rejection is requested.

Rejection Under 35 U.S.C. 103

Claims 1-3, 6-8, and 12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ghannadan et al. (Int Arch Allergy Immunol. 127: 299-307, 2002) in view of Goodell et al. (J. Exp Med, 183: 1797-1806, 1996).

The Examiner alleges that it would have been obvious to the ordinarily skilled artisan to modify the teachings of Ghannadan et al. by utilizing the Hoechst 33342 dye exclusion technology of Goodell in order to obtain a pure enriched population of EPCR+ cells in a biological sample" (page 5 of the Office Action). Applicant respectfully disagrees and traverses the rejection.

The teachings and deficiencies of Ghannadan et al. have been discussed in response to the previous § 102 rejection. Ghannadan et al. does not disclose a method by which a population of hematopoietic stem cells can be obtained. HMC-1 is an immature mast cell line and it would not

have been possible to obtain any hematopoietic stem cells. It also would not have been possible (or obvious) to obtain substantially pure populations of hematopoietic stem cells.

Goodell et al. does not cure the deficiencies of Ghannadan et al. Even if a skilled artisan had combined the teachings of Ghannadan et al. and Goodell et al., it would not have been possible to obtain hematopoietic stem cells, since they are not produced by the HMC-1 cell line. Therefore, the combined references cannot render obvious the claimed invention; the combination of cited references does not yield all the limitations of the claimed invention.

Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

Claims 1-3, 6-8, and 12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ghannadan et al, (Int Arch Allergy Immunol. 127: 299-307, 2002) in view of Miyazato et al, (Blood, 98: 422-427, 2001).

The Examiner alleges that "it would have been obvious to the ordinarily skilled artisan to modify the teachings of Ghannadan et al. by utilizing column chromatography in order to obtain enriched population of EPCR+ cells in a biological sample as taught by Miyazato et al. with a reasonable expectation of success" (page 7 of the Office Action). Applicant respectfully disagrees and traverses the rejection.

The teachings and deficiencies of Ghannadan et al. have been discussed in response to the previous § 102 rejection. Ghannadan et al. does not disclose a method by which a population of hematopoietic stem cells can be obtained. It would not have been possible to obtain hematopoietic stem cells or substantially pure populations of hematopoietic stem cells using the cell line HMC-1, which is an immature mast cell line.

Miyazato et al. does not cure the deficiencies of Ghannadan et al. Even if a skilled artisan had combined the teachings of Ghannadan et al. and Miyazato et al., it would not have been possible to obtain hematopoietic stem cells. The combined references do not render obvious the claimed invention, at least because the combination of cited references cannot yield all the limitations of the claimed invention.

Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

CONCLUSION

A Notice of Allowance is respectfully requested. The Examiner is requested to call the undersigned at the telephone number listed below if this communication does not place the case in condition for allowance.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, the Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith to our Deposit Account No. 23/2825, under Docket No. C1233.70001US01.

Dated: March 31, 2010

Respectfully submitted,

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ESTABLISHMENT OF AN IMMATURE MAST CELL LINE FROM A PATIENT WITH MAST CELL LEUKEMIA*

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Abstract—A cell line showing many characteristics of immature mast cells has been established from the peripheral blood of a patient with mast cell leukemia. Cultured cells contain low levels of histamine, are stained metachromatically by toluidine blue, and contain chloroacetate esterase, aminocaproate esterase and tryptase activities. The cells lack T and B lymphocyte, as well as myeloid cell markers, and do not possess IgE receptors. Solid tumors of metachromatically positive cells have been successfully induced and serially passed in nude mice using 5-azacytidine transformed cells. This cell line may be useful for future studies of mast cells and their constituents.

Key words: Mast cells, mast cell leukemia, chromosomal translocation.

INTRODUCTION

THE STUDY of human mast cells has been hampered, in part, by the difficulty of obtaining sufficient numbers of purified cells. Although mast cells are a normal constituent of human bone marrow [1], they generally are not found in circulation and only small numbers of mast cells are present in agar culture of normal human bone marrow [2]. We report studies on a cell line established from a patient with mast cell leukemia. The cultured cells bear many similarities to immature mast cells, grow in the absence of human plasma or leukocyte-conditioned medium, and possess a unique chromosomal translocation. We have referred to the cell line as human mast cell-1 (HMC-1).

* Supported in part by grants from the National Institutes of Health, AI 20416, AI 15231 and AI 09728, and by the Mayo Foundation.

Abbreviations: *WBC*, white blood cell; *HMC-1*, human mast cells-1; *PBS*, Dulbecco's phosphate buffered saline; *RPMI*, Roswell Park Memorial Institute; *MEM*, Eagle's minimum essential medium; *MBP*, eosinophil granule major basic protein; *CLC*, Charcot–Leyden crystal; *CALLA*, common acute lymphocytic leukemia antigen; *EBNA*, Epstein–Barr nuclear antigen; *TdT*, terminal deoxynucleotidyl transferase; *EDN*, eosinophil-derived neurotoxin; *ECP*, eosinophil cationic protein; *EPO*, eosinophil peroxidase; *Au*, gold; *Pd*, palladium; *IL-3*, interleukin-3; *HLMC*, human lung mast cell.

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MATERIALS AND METHODS

Case report

The clinical aspects of this case are reported elsewhere [3]. A nonatopic 52-yr-old patient with a five-month history of nausea, vomiting, diarrhea, flushing, fatigue and weight loss was admitted to a Mayo Clinic-affiliated hospital. Clinical examination revealed splenomegaly, but was otherwise negative. The hemoglobin was 9.5 g/dl, platelet count 82,000/mm^3, and white blood cell (WBC) count 6500 with a differential count of 54.5% neutrophils, 30.5% lymphocytes, 2.5% monocytes, 0.5% eosinophils, 0.5% metamyelocytes, 1% myelocytes and 10.5% cells which resembled mast cells. Cytochemical studies on peripheral blood mast cells showed that they were peroxidase and nonspecific esterase negative, chloroacetate esterase and toluidine blue positive. A bone marrow aspirate revealed 80% cellularity with sheets and clusters of chloroacetate esterase positive cells. Karyotypic analysis of bone marrow cells was normal. A splenectomy was performed to relieve the thrombocytopenia and anemia. Wedge biopsy of the liver showed modest periportal and sinusoidal infiltration with cells which were positive for chloroacetate esterase and aminocaproate esterase. The spleen was diffusely infiltrated with mast cells, and showed foci of extramedullary hematopoiesis. A diagnosis of mast cell leukemia was made.

During the last two months of life, the patient underwent vagotomy, pyloroplasty and patch repair of a perforated duodenal ulcer. Final admission to the hospital was prompted by recurrent fever of unknown cause. The initial blood count revealed hemoglobin 9.4 g/ml, platelet count 122,000/mm^3 and WBC 39,300 (60.5% abnormal mast cell forms). Multiple cultures were negative. Despite administration of antibiotics, episodes of fever to 40°C continued. Therapy with hydroxyurea, vincristine and Solu Medrol was unsuccessful. The patient succumbed to progressive

respiratory distress, disseminated intravascular coagulation and massive upper gastrointestinal hemorrhage.

Cell culture

During the patient's final admission, after obtaining informed consent, a leukopheresis was performed. An aliquot of this cell suspension was used for cell culture. Cells were diluted 1:6 with sterile Dulbecco's phosphate buffered saline (PBS), and light density cells were obtained by Percoll density gradient separation (1.077). The light density interface cells were washed twice with a large volume of PBS and cultured at 1×10^6/ml in Iscove's medium with 20% defined calf serum (Hyclone Laboratories, Logan, UT) and 1.2 mM alpha thioglycerol. The cultures were incubated at 5% CO_2 and 37°C in a saturated water vapor atmosphere. Once a week the cultures were fed by removal and replacement of half of the culture medium. Cell counts and viability testing were done at these times and viable cell numbers adjusted to 0.5×10^6–1×10^6 cells/ml.

Variations in culture conditions were tested to optimize cellular growth. First, the requirement for leukocyte conditioned medium was assessed. Briefly, 10% leukocyte conditioned medium (prepared by incubating human buffy coat cells in Iscove's medium with 1% phytohemagglutinin, 10% serum for 1 week) was incubated with cells, and growth was compared to cells cultured in the absence of leukocyte conditioned medium. Cell counts and viability were examined at days 2, 4, 6 and 8. Second, the necessity for defined calf serum was examined by varying the concentration from 0 to 20% and evaluating cell numbers and viability (on days 3 and 7). Third, the optimal cell culture medium was determined by utilizing Roswell Park Memorial Institute (RPMI) or Eagle's minimum essential medium (MEM) in lieu of Iscove's medium. Cell numbers and viability were determined on day 7. Lastly, the effect of interleukins was tested by thymidine incorporation on days 2, 4, 6 and 8 after incubation of cells (1×10^4 cells/well in Iscove's medium, 10% defined calf serum, 1.2 mM alpha thioglycerol) with either recombinant murine interleukin-3 (IL-3) (final concentration 10–10,000 U/ml) or with control (COS 7 cell line) conditioned medium, both kindly provided by DNAX (Palo Alto, CA). Replicate microtiter wells (minimum of 5) were counted at each date.

Cytochemical staining

Cytochemical staining properties of cultured cells were examined with the following stains: Wright–Giemsa [4], toluidine blue [5], alcian blue [6], Luxol-fast blue [7], napthol AS-D chloroacetate esterase [8], cyanide-resistant eosinophil peroxidase [9], aminocaproate esterase [10], eosinophil major basic protein (MBP) [11], Charcot–Leyden crystal (CLC) protein [12], and conjugated avidin [13]. Cytochemical staining of cells for mast cell tryptase and chymotryptic proteinase were kindly performed by Dr L. Schwartz [14].

Expansion of the HMC-1 cell line

To obtain enhanced metachromasia, cells were cloned by plating at 1×10^4/ml in 0.9% methylcellulose, 4.5×10^{-5} M 2-mercaptoethanol and 30% defined calf serum at 5% CO_2 in a saturated water vapor atmosphere. After 8 days, colonies were randomly aspirated using a finely-drawn sterile microcapillary. Colonies were transferred to 0.2 ml Iscove's medium, 1.2 mM alpha thioglycerol, 10% defined calf serum, in a Falcon 96-well microtiter plate, and subsequently transferred to 24-well culture plates and to 25-cm^2 tissue culture flasks. Cultured cells were examined for metachromatic staining and fed weekly.

Cells were also cultured in the presence of 5-azacytidine to induce differentiation. Cells at 1×10^6/ml in Iscove's medium containing 20% defined calf serum, and 1.2 mM alpha thioglycerol were incubated with 5-azacytidine [15] (final concentration 3 micromolar) for 7 days after which cells were washed and cultured in Iscove's medium containing 10% defined calf serum and 1.2 mM alpha thioglycerol. The percentage of metachromatically staining cells was determined by staining with acidic toluidine blue. Cells with enhanced metachromatic staining and stable growth properties were saved for further study.

To determine if 5-azacytidine-derived cells could successfully be propagated *in vivo*, nude mice were given intramuscular injections of 10 to 20×10^6 cells. The recipient animals were observed for tumor growth for several months.

Cytogenetic studies

Cytogenetic studies were performed on bone marrow cells and splenocytes obtained antemortem, cells obtained by leukopheresis, and cells obtained from *in-vitro* cultures. Following short-term (24–48 h) tissue culture in medium without mitogens, slide preparations were made using colcemid, hypotonic solution and a methanol-glacial acetic acid fixative [16, 17]. Metaphases were analysed using GTG- and QFQ-banding.

Cell surface markers

Cultured cells were examined for the presence of membrane antigens using a cytochemical technique employing immunoperoxidase [18, 19]. Monoclonal antibodies to the following markers were utilized: Leu 14, Leu 4, Leu 7, Leu M1 (OKM1), HLA-Dr, OKT4, OKT8, LYT3, HP1-1D and common acute lymphocytic leukemia antigen (CALLA). Epstein–Barr nuclear antigen (EBNA) was assayed by the method of Reedman and Klein [20]. Cells were examined for terminal deoxynucleotidyl transferase (TdT) by the method of Hecht *et al.* [21].

Mediator content

Histamine content of cultured cells was determined after 10 freeze–thaw cycles by a double isotope technique [22] described previously. MBP content was determined by radioimmunoassay [23] after lysis of cells with Nonidet P40. In addition, eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) were measured by radioimmunoassay as previously described [24]. Eosinophil peroxidase (EPO) was measured by a newly developed competitive binding radioimmunoassay (unpublished observation).

Determination of IgE binding

Cultured cells were tested for the presence of IgE receptors by the method of Conrad *et al.* [25] utilizing ^{125}I-labeled IgE, and by a rosette assay employing ox erythrocytes coated with human IgE [26]. As a positive control, human B lymphoblastoid cells (RPMI 8866) with low affinity IgE receptors were used [25]. Rosettes were defined as cells having three or more adherent erythrocytes.

Scanning electron microscopy

Plastic-adherent cells, cultured in the absence of serum,

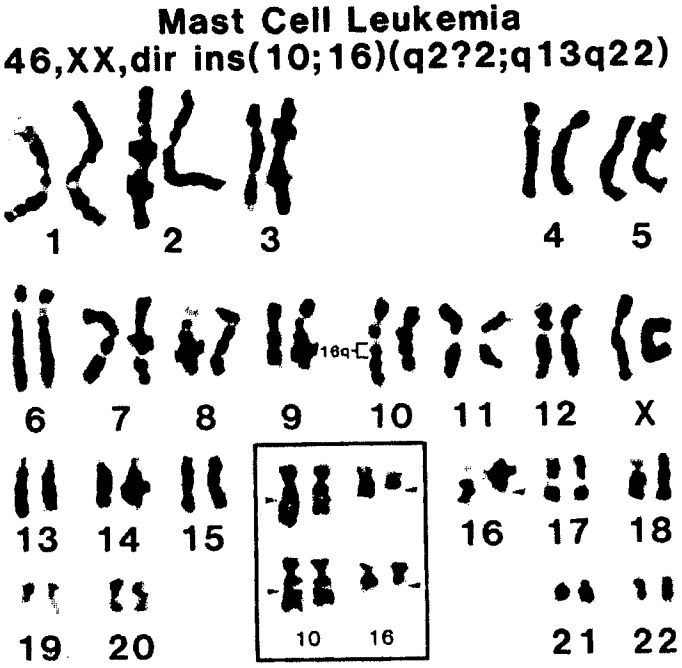

FIG. 1. Representative GTG-banded karyotype of HMC-1 cells. The translocation of part of the long arm of chromosome 16 into the long arm of a chromosome 10 is seen. The inset shows this same translocation in the number 10 and 16 chromosomes from two other metaphases.

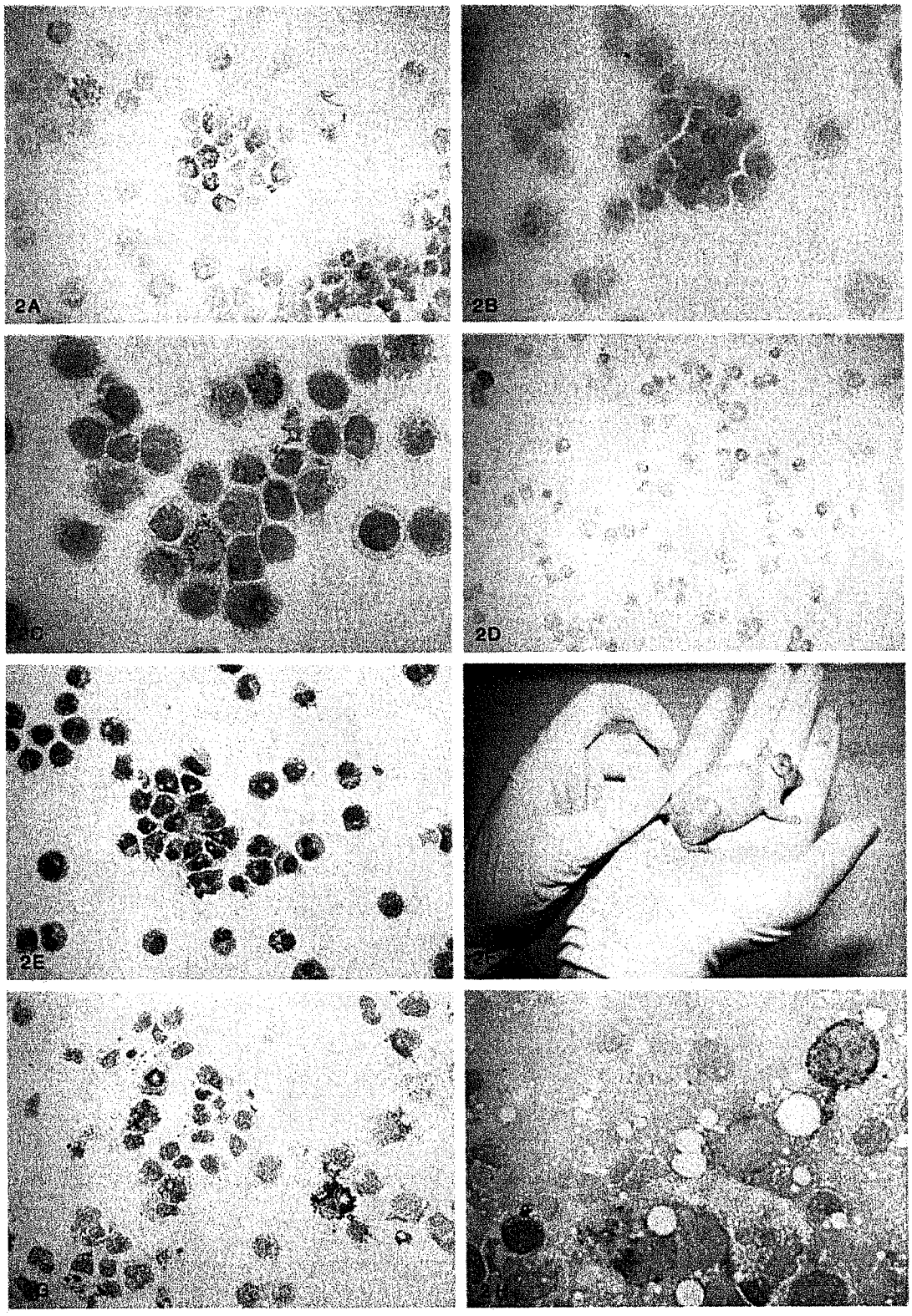

FIG. 2. Cytochemical stains of HMC-1 cells from suspension culture and of an HMC-1 cell-derived mastocytoma from a nude mouse. (A) Acidic toluidine blue stain of HMC-1 clone A_4 demonstrating variable metachromatic staining (original magnification × 400). (B) Chloroacetate esterase stain of HMC-1 cells demonstrates intense granular staining in several cells (original magnification × 630). (C) Aminocaproate esterase stain of HMC-1 clone A_4 reveals positive, though variable, staining in numerous cells (original magnification × 630). (D) Alcian blue/safranin stain of HMC-1 clone C_2 demonstrating blue cytoplasmic staining (original magnification × 100). (E) Wright–Giemsa stain of HMC-1 clone A_4 shows variably granulated cells with differing nuclear and cytoplasmic morphology (original magnification × 400). (F) Initial solid tumor which developed in the hind limb of a nude mouse 3 months after an injection of 10×10^6 cells from the 5-azacytidine-derived HMC-1 clone. (G) Acidic toluidine blue staining of cells comprising the solid tumor illustrated in (F) (original magnification × 630). (H) Positive aminocaproate esterase stain of cells comprising the solid tumor illustrated in (F) (original magnification × 1000).

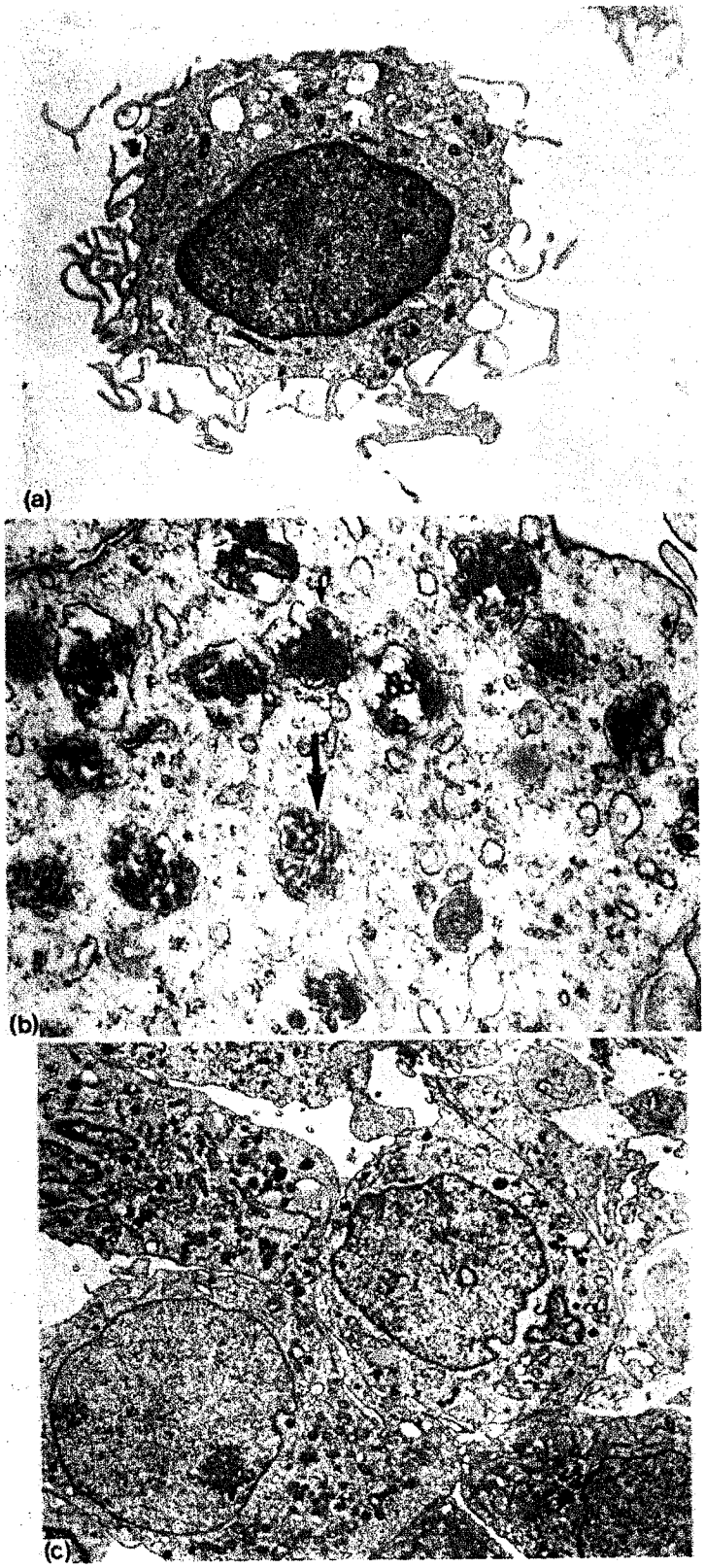

FIG. 3. Electron micrographs of cultured HMC-1 cells and of an HMC-1 derived mastocytoma from a nude mouse. (a) Electron micrograph of a mast cell from suspension culture shows immature granules and a central round nucleus with dispersed nuclear chromatin. Numerous cytoplasmic processes are present (× 3600). (b) Higher magnification views of another suspension culture-derived cell shows the variability of granule morphology. Some granules consist of an electron-dense material (small arrow) while in other granules small whorls and cylindrical structures can be seen (large arrow) (× 16,200). (c) Electron micrograph of cells from a solid tumor of metachromatically positive cells which arose in a nude mouse given an intramuscular injection of 5-azacytidine transformed HMC-1 cells. Numerous mitochondria, lipid bodies, small granules, and large central ovoid nuclei containing dispersed chromatin can be seen (× 2850).

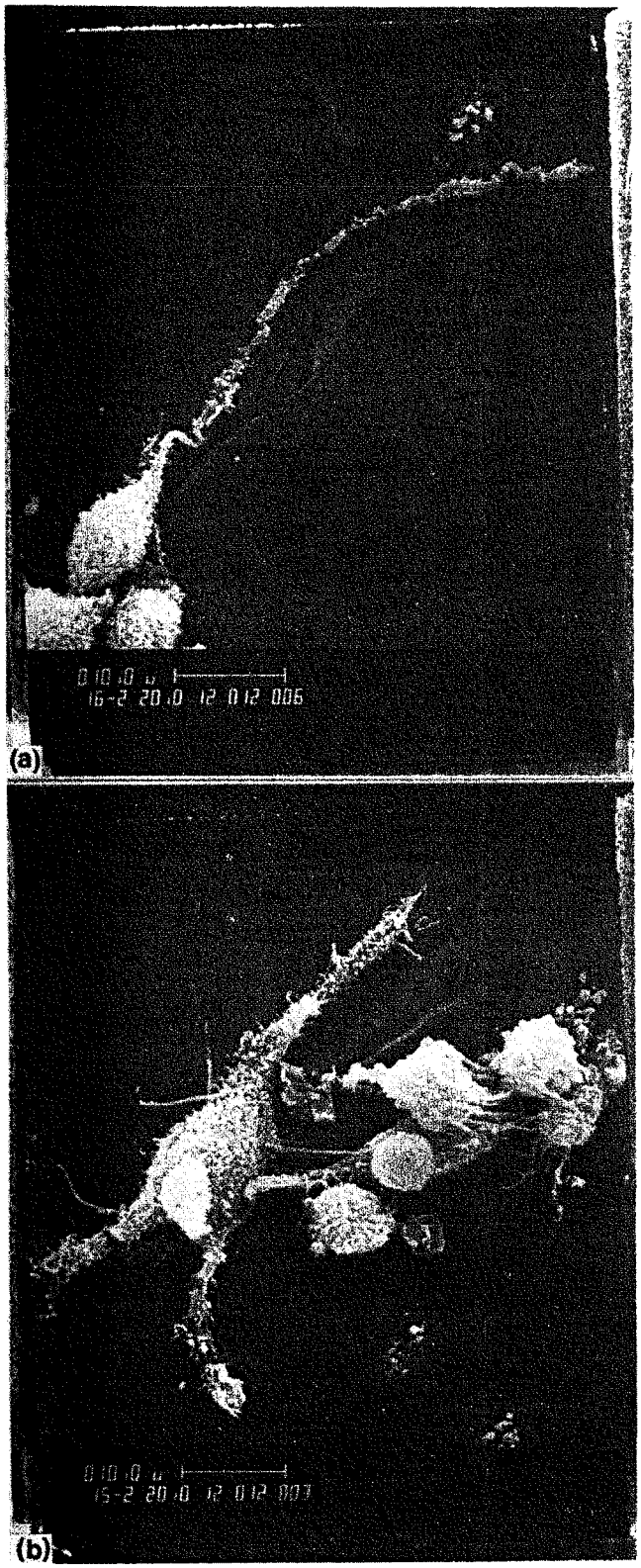

FIG. 4. Scanning electron microscopy of HMC-1 cells grown in the absence of serum. HMC-1 cells often assumed a flattened, irregular shape and adhered to plastic when cultured without serum. Cytoplasmic processes, some of which were extremely long (a), appeared to make contact with neighboring cells (b) (original magnification: (a) × 1600, (b) × 1500).

were examined by scanning electron microscopy. Briefly, plastic coverslips were placed on the bottom of tissue culture dishes and removed following a 4-day culture. Coverslips were fixed in Trump's fixative, rinsed and dehydrated through graded alcohol solutions. Samples were then loaded in precleaned BEEM capsules (Better Equipment for Electron Microscopy Inc., Tristin, CA) and reimmersed in absolute alcohol. Following critical point drying, specimens were mounted on aluminium stubs and sputter coated with gold and palladium (Au/Pd) for routine scanning electron microscopy.

Transmission electron microscopy

Cells from suspension culture were prepared for electron microscopy by the method of Caulfield [27], with slight modifications. Briefly, cells were fixed in ice-cold Karnovsky's solution for 10 min [27]. After three rinses in cacodylate buffer pH 7.4, cells were postfixed in 1% OsO_4 for 1 h on ice, rinsed three times in distilled water, and en bloc stained in 2% uranyl acetate for 2 h at room temperature. Following dehydration through an alcohol series, cells were embedded and sectioned in Spurr low viscosity epoxy resin (Electron Microscopy Sciences, Ft Washington, PA) or in araldite resin grade 502 (Polysciences, Warrington, PA). A section of a mastocytoma from a nude mouse was fixed in Karnovsky's solution for 1 h at 4°C and thereafter processed as described above.

RESULTS

Growth of cells in vitro

By two weeks after initiation of the cultures, viable cell numbers had decreased by approx. 50%. Thereafter, a slow but progressive increase in cell numbers occurred. The original cell density (1×10^6 cells/ml) was not reached for approximately two months. Thereafter, cell numbers and viability were examined weekly and cell density was adjusted to 0.5×10^6 cells/ml.

After feeding, a several-day lag period was often noted before an increase in cell numbers occurred. Thereafter, cell division proceeded irregularly. A plateauing of cell numbers at $1.0–1.4 \times 10^6$ cells/ml was generally reached at day 6–8. Cultured cells formed clumps and aggregates in suspension culture making exact cell quantitation difficult. Leukocyte conditioned medium did not enhance viable cell numbers; however, both cell numbers and viability increased in the presence of 10 or 20% defined calf serum. In the absence of calf serum, cell growth was minimal and cells adhered to plastic. Both RPMI and MEM were inferior to Iscove's medium in their support of cell growth. Incubation of cells with murine IL-3 for 2–8 days did not increase thymidine incorporation above control values.

Cytogenetics

Cytogenetic analysis of 20 bone marrow cells and 8 spleen cells, obtained antemortem, revealed only cells with a normal 46,XX karyotype. However, 20 cells examined from this cell line after three months in culture each had a 10;16 translocation (Fig. 1). Therefore, we examined frozen cells obtained at the time of leukopheresis; although only four metaphases were seen, each showed the same chromosomal change observed in the cell line. Thus, the clone of cells with a 10;16 translocation was present in the patient before death and not induced by *in-vitro* culture conditions. A second study involving analysis of 33 metaphases from the cell line after seven months of culture revealed that the karyotype of the cells still had the 10;16 translocation and that no other abnormalities had developed.

Cytochemical and biochemical properties

As shown in Fig. 2, cultured cells stained positive with the following cytochemical stains: toluidine blue, alcian blue, chloroacetate esterase and aminocaproate esterase. Wright–Giemsa stain revealed variably granulated cells. Immunofluorescent staining for eosinophil MBP was also positive (not shown). Cytochemical staining for mast cell tryptase revealed 5.2% positively staining cells. Two sublines established from HMC-1 cells cloned in methylcellulose also showed that 6.3 and 10.5% of cells were stained with anti-tryptase antibody. Cultured cells did not stain with anti-chymotryptic proteinase antibody. Less than 1% of cells stained positively with conjugated avidin. Stains with Luxol-fast blue and for cyanide-resistant peroxidase and CLC protein were negative.

Cultured cells were negative to B cell, T cell and myeloid cell surface markers, as well as for EBNA, CALLA and TdT. Histamine content of HMC-1 cells and of several clones was <1 pg/cell. Cultured cells contained measurable quantities of eosinophil MBP and EPO; EDN and ECP were not detectable (Table 1). HMC-1 cells, as well as cells obtained directly from leukopheresis and from the Percoll 1.077 interface prior to culture, failed to bind ^{125}I-IgE. In parallel assays, RPMI 8866 cells bound low levels of ^{125}I-IgE, which could be inhibited by preincubation with

TABLE 1. MEDIATOR CONTENT OF HMC-1 CELLS*

Protein	ng/10^6 cells
Eosinophil major basic protein (MBP)	506
Eosinophil peroxidase (EPO)	41
Eosinophil-derived neurotoxin (EDN)	<3
Eosinophil cationic protein (ECP)	<1

* HMC-1 cells cultured in 20% serum.

Comparable values for eosinophil proteins/10^6 eosinophils are: MBP—6000 ng; EPO—7500 ng; EDN—6600 ng; ECP—2100 ng [60].

cold IgE in 50- to 100-fold excess. Cultured cells did not form rosettes with IgE coated ox erythrocytes.

Electron microscopy

The ultrastructural appearance of cultured cells was similar to that of immature cloned murine mast cells [28]. Cells typically had a round or ovoid central nucleus, immature granules and cytoplasmic processes (Fig. 3a). Granule morphology was variable (Fig. 3b). Some granules consisted primarily of an electron-dense material (small arrow), while other granules contained small scroll-like or tubular structures (large arrow). Compared to cells from suspension culture, the appearance of HMC-1 cells growing as solid tumors in nude mice was notable for better preservation of granule matrices and less cytoplasmic vacuolation. Numerous small granules and lipid bodies were present (Fig. 3c).

Scanning electron microscopy revealed the adherent cell population consisted of cells of various sizes having one or more cytoplasmic processes (Fig. 4a). These processes, which were often quite elongated, frequently appeared to make contact with neighboring cells (Fig. 4b).

HMC-1 clones and cell propagation in nude mice

Several mast cell clones were established. The percentage of metachromatically staining cells in these clones ranged from 16 to 66%. Histamine content of HMC-1 clones was also less than 1 pg/cell. Of interest, cells incubated with 5-azacytidine subsequently developed an enhanced replicate ability showing a doubling time of three to four days compared with six to eight days for uncloned HMC-1 cells. We have maintained these cells as separate lines for over one year. Three months following intramuscular injection of one of the 5-azacytidine-derived lines a solid tumor developed in the hind limb of a nude mouse (Fig. 2F). Toluidine blue staining of touch preparations made from the cut surface of the tumor or of cytospin preparations of dispersed cells revealed cells with variable metachromatic staining (Fig. 2G) which were also positive for aminocaproate esterase activity (Fig. 2H) and chloroacetate esterase activity (not shown). Serial passage of this cell line has been accomplished by placing small tumor explants subcutaneously in recipient nude mice. New tumors (1–2 cm^3 size) arise every 8–12 weeks. Individual tumors can be removed from a recipient mouse while several other tumors are allowed to continue to grow on the same animal.

DISCUSSION

We report initial studies on an immature mast cell line, which we refer to as HMC-1, derived from a patient with mast cell leukemia. HMC-1 cells grow best in the presence of defined bovine calf serum but require no autologous serum or conditioned medium.* Similar to the cell growth pattern reported for cultured human mastocytosis-derived mast cells [30], HMC-1 cells formed clumps and small aggregates in suspension culture. Cellular adherence to plastic occurred when cells were grown in serum-free culture medium. The contents of HMC-1 granules may be a result of the undifferentiated state of these cells, their leukemic origin, and/or the presence of a chromosomal rearrangement. The selective presence of MBP (and absence of EDN or ECP) in HMC-1 cells was unexpected because this protein has not been found in connective tissue mast cells [31]. However, recent analyses of human tissues suggest that a population of mast cells may either synthesize or endocytose MBP [32]. HMC-1 cells stained positively with acidic toluidine blue and alcian blue stains. Stains for aminocaproate esterase and chloroacetate esterase activity were also positive, indicating that HMC-1 cells are related to mast cells and not to basophils or immature neutrophils [3, 33–35]. Tryptase activity, specific for mast cells, was also present in HMC-1 cells and in clones derived from them. Selective localization of tryptase activity to mast cells has been reported [36, 37]. Thus, these cells possess numerous enzymes characteristic of and confined to mast cells.

Electron microscopic appearance revealed immature cells with granules whose structure only rarely resembled that of mature mast cells [38–40]. Scanning electron microscopy showed that HMC-1 cells possessed elongated cytoplasmic processes. In previous reports, leukemic mast cells have been found to have elongated cytoplasmic "tails" [41, 42].

Examination of HMC-1 cells for B cell, T cell and myeloid surface antigens was negative. HMC-1 cells did not bind ^{125}I-IgE or form rosettes with IgE-coated ox erythrocytes. In previous studies human mastocytoma cells cultured for up to 30 days had been reported to maintain IgE receptors [43] as had cloned murine mast cells [44]. IgE receptors have been found on human basophils [45, 46], and direct evidence for IgE receptors on human mast cells has come from studies of dispersed human lung mast cells (HLMC) [47–49] and partially purified populations of intestinal mast cells [38]. However, other studies

* Temporarily successful *in-vitro* culture of histamine-containing cells from another patient with mast cell leukemia has been reported [29]; however, cell growth was absolutely dependent on the presence of autologous serum.

suggest that certain mast cell populations may lack IgE receptors. In one report, alcian blue positive mucosal mast cells lacking IgE were identified in *Trichinella spiralis* infected mice [50]. In a second report, human leukemic mast cells failed to show membrane-bound IgE [42]. Lastly, an impaired secretory response to anti-IgE antibody was found in another case of mast cell leukemia [51]. Thus, immature mast cells such as those which circulate in cases of mast cell leukemia may have lost the ability to synthesize IgE receptors or synthesize abnormal receptors which do not bind IgE.

HMC-1 cells possess a 10;16 translocation—46,XX,dir ins(10;16) (q2?2;q13,q22). This chromosomal change was present in cells initially collected by leukopheresis, indicating that this anomaly was not an artifact of culture. Thus, this translocation is evidence that this cell line was actually derived from a chromosomally abnormal clone that was present in the patient. This translocation was not observed in bone marrow and spleen samples collected several months prior to this patient's death. However, we may not have detected this abnormality because of sampling error by inadvertently examining only normal cells.

Mast cell clones having enhanced metachromatic staining were established from the original cell line. Of special interest, HMC-1 cells incubated with 5-azacytidine developed accelerated proliferative activity. Three months after injection into a nude mouse these cells established a solid tumor comprised of metachromatically positive cells, which were also positive for chloroacetate esterase activity, and which had ultrastructural features of immature mast cells. Serial passage of this solid tumor has been successful.

The bone marrow origin of human mast cells [43] has been suggested by the finding of hemopoietic tissue-specific antigens on mast cells cultured from the bone marrow of a patient having systemic mastocytosis. Despite the fact that HLMC have been purified, establishment of independent cell lines with HLMC has not been reported [52]. A common progenitor for mast cells and basophils may exist because mast cell and basophil precursors have been found among the blast cells in chronic granulocytic leukemia [53–56]. Also, clonal origin of human "basophil/mast" cells from circulating multipotent hematopoietic progenitors has been shown by combined use of histamine and G6PD isoenzyme analysis of single mixed hematopoietic colonies containing granulocytic, erythrocytic, macrophage-monocytic and megakaryocytic cells [57]. Similarly, "mast cells/basophils" can be grown in suspension culture of umbilical cord mononuclear cells [58] or from the peripheral blood of allergic volunteers [59]. To our knowledge, however, successful long-term culture of autonomously growing immature human leukemic mast cells has not previously been reported. HMC-1 cells may prove useful for study of mast cells and their constituents.

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